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4/6/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: MARSHALL, et al.  
SERIAL NO: 09/193,653  
FILED: November 17, 1998  
TITLE: METHODS AND COMPOSITIONS FOR MODULATING  
IMMUNE SYSTEMS OF ANIMALS

GRP./A.U.: 1645  
EXAMINER: R. Zeman  
DOCKET NO: P01936US4

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132 DECLARATION OF WILLIAM E. MARSHALL

Assistant Commissioner for Patents  
Washington, D.C. 20231

**132 DECLARATION OF WILLIAM E. MARSHALL**

Dear Sir:

I, William E. Marshall hereby declare the following.

1. I am the inventor on the above-identified case and am familiar with the prosecution including the office action dated July 10, 1997.

2. My background includes a Ph.D. in biochemistry from the University of Illinois, post-doctoral training at Uppsala University and Cambridge University, assistant professor of biochemistry at the University of Minnesota, director of technology development at General Foods Corp., president of the Microbial Genetics Division of Pioneer Hi-Bred International, member of the Iowa Academy of Sciences, chairman of the National Agricultural Research and Extension Users Advisory Board of the U.S. Congress, member of the advisory panel on biotechnology to the Office of Technology Assessment of the U.S. Congress, member of the advisory panel on intellectual property to the GATT, and associate professor of microbiology and immunology at the New York Medical College.

3. I understand that the Examiner has placed rejections on my application based upon one or more of the following references: Perdigon et al 1990 J Food Production

53 (5) p. 404-410; De Vuyst et al 1996 142 p. 817-827; Nanji U.S. Patent 5,413,785; Farr U.S. Patent 3,953,609 and Emery U.S. Patent 5,538,733. This application details scientific argument and experimental evidence to refute the Examiner's contentions.

4. Our invention is not limited to only stressing lactic acid bacteria. We have demonstrated that the phenomenon of stressed bacteria releasing immune-activating SRFs <10 kDa is also observed in gram-negative bacteria, *S. typhimurium*, *E. coli*, and *K. pneumoniae*, a strict anaerobe, *B. coryneforme*, and the gram-positive pathogens, *S. aureus*, *L. monocytogenes*, and *S. pyogenes*. The use of harmless generally regarded as safe lactic acid bacteria has been emphasized only for purposes of minimizing manufacturing costs and allowing the technology to be practiced as a cottage industry.

5. SRFs are a mixture of compounds having an absorption maximum at 254 nm, indicating the presence of nucleotides, not proteins. Proteins have a maximum at 280 nm.

6. Attached herewith and for the Examiner's consideration are 4 photographs obtained from experiments conducted to refute several of the Examiner's contentions.

The protocols are detailed beneath the photos. The results show that neither total SRFs nor SRFs <10 kDa prepared from stressing 5 strains of *Lactobacillus plantarum*, 2 strains of *Enterococcus faecium*, *L. caseii*, *L. acidophilus*, and *Listeria monocytogenes* contain bacteriocins. The release of SRFs was induced by transferring them from their growth media into Dulbecco's LPS-free phosphate-buffered-saline, pH 7.3 for 20 hours, at 37°C unless specified otherwise.

**Figure 1** is a photo demonstrating that neither total SRFs nor those <10 kDa released by stressing bacteria results in the production of bacteriocins.

**Figure 2** is a photo demonstrating that stressing higher levels of bacteria, or stressing bacteria twice or heat-killing bacteria does not result in the production of bacteriocins.

**Figure 3** is a photo demonstrating that neither total SRFs nor those <10 kDa released by stressing bacteria results in the production of bacteriocins.

**Figure 4** is a photo demonstrating that neither total SRFs nor those <10 kDa released by stressing bacteria results in the production of bacteriocins tested against a lawn of a mixture of lactic-acid bacteria.

7. The following experiments were conducted which show that lactic-acid bacteria do not inhibit the growth of *E. coli*. To determine whether viable lactobacilli inhibit the growth of undesirable Gram-negative bacteria, a pour plate of  $10^6$  CFUs per ml. of *E. coli* in Minimal Media + 0.1% dextrose was prepared.

At the asterisk " 10 $\mu$ L of 25.  $\mu$ g of Nisin were added as a positive control.

At spot "1", 10 $\mu$ L aliquots of  $10^9$  CFUs of viable *L. caseii* were added.

At spot "2", 10 $\mu$ L aliquots of  $10^9$  CFUs of viable *L. fermentum* were added.

At spot "3", 10 $\mu$ L aliquots of  $10^8$  CFUs of viable *L. acidophilus* were added.

Slight inhibition of the growth of *E. coli* was seen only around Nisin. The test strains did not inhibit the growth of *E. coli*. The photo is not included; its definition was insufficient to show the zone around Nisin on the background of Minimal Medium.

8. Nanji teaches the use of a unique lactobacillus that is acid-resistant and able to colonize over-crowded,

epithelial mats of bacteria and destroy endotoxin-producing Gram-negative bacteria that may be present. He teaches that blood endotoxin levels can be reduced by feeding  $10^{8-14}$  CFUs per day of a unique *Lactobacillus* GG. He teaches that reduced levels of blood endotoxin reflect the destruction of Gram-negative organisms in the gut by the over-growth of the lactobacillus.

Nanji does not show that an ordinary lactobacillus would not provide the blood LPS-reducing benefits. Nor does he demonstrate the *E. coli* - inhibiting activity of *Lactobacillus* GG. As indicated earlier, we have demonstrated that SRF-releasing lactobacilli do not inhibit the growth of endotoxin-producing *E. coli* in lab media, but they do, however, release SRFs that protect mice against the lethality of an LPS injection.

9. Perdigon teaches that the health benefits of feeding milk fermented with lactobacilli is due to an interaction between the bacteria and the milk solids.

In other words, she states that the immune-enhancing effect of the lactobacilli was due to products created by lactobacilli fermenting milk proteins and the action of milk-derived lysozyme on bacteria already established in the gut. The observations of Perdigon would not lead one of

skill in the art to think in terms of stressing bacteria and collecting the released low molecular weight products. On the contrary, one would use milk in all experiments. Our application teaches that the origin of SRFs is the bacteria themselves responding to being transferred to a new environment, not the products of their media.

10. Emery teaches the use of a subcutaneous implant that slowly releases an immunogen to selectively induce the formation of specific antibodies. We do not teach implanting, or slow release or the use of immunogens >30kDa fractions, or the formation of specific antibodies. The reference does refer to bacterial SRPs, (column 13, line 60), which are Siderophore-Receptor-Proteins, not Stress-Release-Factors. Siderophore-Receptor-Proteins are bacterial surface receptors that are conserved antigens found on the surfaces of many species of bacteria. They function to sequester iron after binding to surface proteins. Other than a close similarity in the initials, there is no relationship between the two inventions.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of

Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Date: 3-6-01

William E. Marshall  
William E. Marshall



Figure 1

Bacteriocins are not produced by stressing  $10^9$  CFUs per ml. of *L. monocytogenes*, *L. plantarum* or *E. faecium*

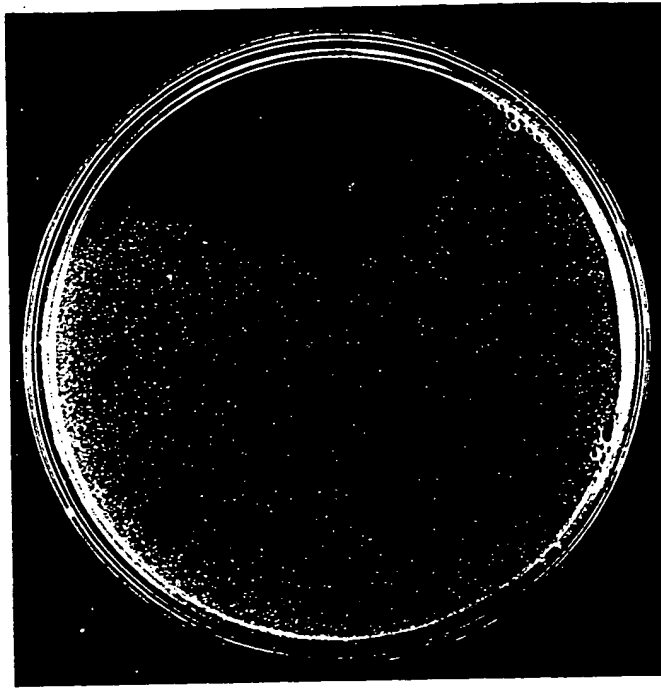


Figure 1 is a photo showing the results of an assay of various preparations of SRFs to inhibit the growth of the test strain, *Lactobacillus helveticus*, ATCC 15009. A lawn of  $10^6$  CFUs of *L. helveticus* was pour-plated in MRS agar and allowed to stand for 3 hrs. before SRFs were added and the plate inoculated at  $37^\circ\text{C}$ .

At the asterisk \*, As a positive control, 25.  $\mu\text{g}$  of the bacteriocin Nisin, purchased from Sigma- Aldrich Co., was added in a  $10\mu\text{L}$  aliquot to the spot at the top marked by an asterisk.. A clear zone can be seen indicating inhibition of the growth of *L. helveticus* due to the bacteriocin, Nisin.

At position "1",  $10\mu\text{L}$  of total SRFs prepared from  $10^9$  CFUs of *L. monocytogenes* per ml were applied. No clear zone of inhibition occurred.

At position "2",  $10\mu\text{L}$  of  $<10$  kDa SRFs prepared from  $10^9$  CFUs of *L. monocytogenes* per ml were applied. No clear zone of inhibition occurred.

At position "3",  $10\mu\text{L}$  of  $<10$  kDa SRFs prepared by stressing, at pH 4,  $10^9$  CFUs per ml of a mixture of 5 strains of *L. plantarum* and 2 strains of *E. faecium* were applied. No clear zones appeared.

Figure 2

Bacteriocins are not produced by stressing  $10^{10}$  CFUs per ml. of *L. monocytogenes* or by twice stressing  $10^9$  CFUs per ml. of *L. plantarum* and *E. faecium* or by stressing heat-killed *L. plantarum* and *E. faecium*



Figure 2 is a photo showing the results of assays to determine if bacteriocins occur in SRF preparations inhibiting the growth of the test strain, *L. helveticus*.

At the asterisk \*, Nisin, the commercial bacteriocin, as a positive control.

At position "4", 10  $\mu$ L of < 10 kDa SRFs prepared from  $10^{10}$  CFUs of *L. monocytogenes* per ml were applied. No clear zones of inhibition occurred.

At position "5", 10  $\mu$ L of <10 kDa SRFs prepared from stressing  $10^9$  CFUs of a mixture consisting of 5 different strains of *L. plantarum* and 2 different strains of *E. faecium* per ml first at pH 4 for 3 hrs. followed by a subsequent stress at pH 7 for 12 hrs. No clear zones of inhibition occurred.

At position "6", 10  $\mu$ L of <10 kDa SRFs prepared from  $10^9$  CFUs of a heat-killed mixture consisting of 5 strains of *L. plantarum* and 2 strains of *E. faecium* per ml were applied. No clear zones of inhibition occurred.

Figure 3

Bacteriocins against the test strain, *L. helveticus* are not produced in the total SRF fraction or in the <10 kDa SRF fraction by stressing  $10^9$  CFUs per ml. of *L. casei*, *L. plantarum* or *E. faecium*.

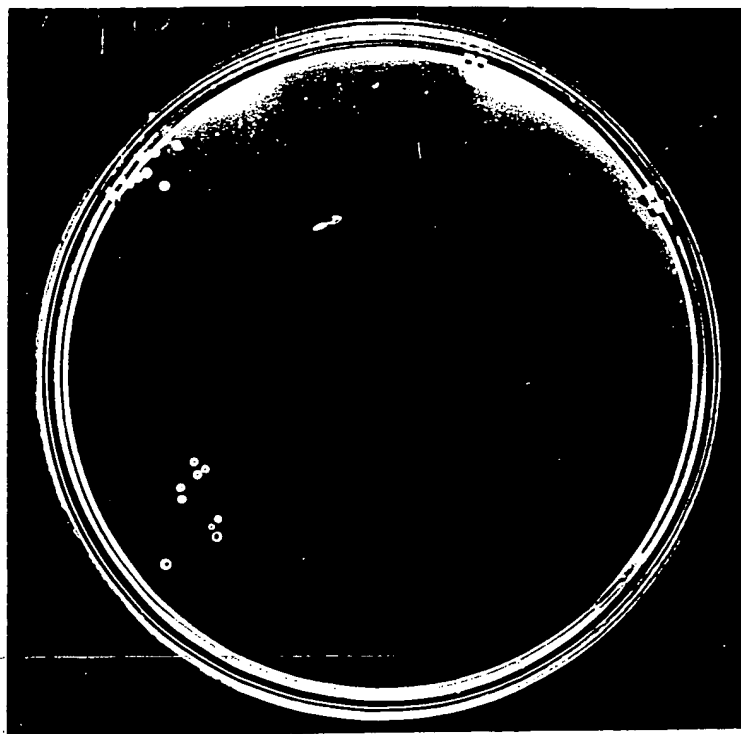


Figure 3 is a photo showing the results of additional assays of SRFs to inhibit the growth of the test strain, *L. helveticus*.

At the asterisk \* : As a positive control, 25.  $\mu$ g of the bacteriocin Nisin, shows a clear zone indicating inhibition of the growth of *L. helveticus*.

At position "1", 10  $\mu$ L of total SRFs prepared from  $10^9$  CFUs of *L. casei* per ml were applied. No clear zone of inhibition occurred.

At position "2", 10  $\mu$ L of <10 kDa SRFs prepared from  $10^9$  CFUs of *L. casei* per ml were applied. No clear zone of inhibition occurred.

At position "3", 10  $\mu$ L of total SRFs prepared from  $10^9$  CFUs of a mixture consisting of 5 different strains of *L. plantarum* and 2 different strains of *E. faecium* per ml were applied. No clear zone of inhibition occurred.

At position "4", 10  $\mu$ L of <10 kDa SRFs prepared from  $10^9$  CFUs of a mixture consisting of 5 different strains of *L. plantarum* and 2 different strains of *E. faecium* per ml were applied. No clear zone of inhibition occurred.

Figure 4

Bacteriocins against 7 test strains of *L. plantarum* and *E. faecium* are not produced in the total SRF fraction or in the <10 kDa SRF fraction by stressing  $10^9$  CFUs per ml. of *L. casei*, *L. plantarum* or *E. faecium*.



Figure 4 is a photo showing the results of an assay for bacteriocins using a mixture of 5 different strains of *L. plantarum* and 2 different strains of *E. faecium* as both SRF-generating strains and as test strains.

At the asterisk \* : As a positive control, 25.  $\mu$ g of Nisin shows a clear zone indicating inhibition of the growth of all the 5 different strains of *L. plantarum* and 2 different strains of *E. faecium*.

At position "1", 10  $\mu$ L of total SRFs prepared from  $10^9$  CFUs of *L. casei* per ml were applied. No clear zones of inhibition occurred.

At position "2", 10  $\mu$ L of <10 kDa SRFs prepared from  $10^9$  CFUs of *L. casei* per ml were applied. No clear zones of inhibition occurred.

At position "3", 10  $\mu$ L of total SRFs prepared from stressing  $10^9$  CFUs per ml of a mixture of 5 strains of *L. plantarum* and 2 strains of *E. faecium* were applied. No clear zones of inhibition occurred.

At position "4", 10  $\mu$ L of < 10 kDa SRFs prepared from stressing  $10^9$  CFUs per ml of a mixture of 5 strains of *L. plantarum* and 2 strains of *E. faecium* were applied. No clear zones of inhibition occurred.